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<b>(21) International Application Number:</b> PCT/US99/23395 <b>(22) International Filing Date:</b> 07 October 1999 (07.10.1999) <b>(30) Priority Data:</b> 60/103,385 07 October 1998 (07.10.1998) US 60/105,805 27 October 1998 (27.10.1998) US 60/116,409 19 January 1999 (19.01.1999) US <b>(60) Parent Application or Grant</b> THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ARKANSAS [/]; (). MANOLAGAS, Stavros, C. [/]; (). JILKA, Robert, L. [/]; (). WEINSTEIN, Robert, S. [/]; (). BELLIDO, Teresita [/]; (). KNOWLES, Sherry, M. ; ().	<b>Published</b>	
<b>(54) Title: IN VITRO AND IN VIVO MODELS FOR SCREENING COMPOUNDS TO PREVENT GLUCOCORTICOID-INDUCED BONE DESTRUCTION</b> <b>(54) Titre: MODELES IN VITRO ET IN VIVO POUR LA DETECTION DE COMPOSES PERMETTANT DE PREVENIR LA DESTRUCTION OSSEUSE INDUITE PAR LES GLUCOCORTICOIDES</b>		
<b>(57) Abstract</b> <p>The present invention demonstrates that glucocorticoid-induced bone disease is due to changes in the birth and death rate of bone cells using a murine model of glucocorticoid excess as well as bone biopsy specimens obtained from patients with glucocorticoid-induced osteoporosis. This invention demonstrates that glucocorticoid administration increases apoptosis of mature osteoblasts and osteocytes and decreases bone formation rate and bone mineral density accompanied by defective osteoblastogenesis and osteoclastogenesis in the bone marrow.</p>		
<b>(57) Abrégé</b> <p>La présente invention vise à démontrer que la destruction osseuse induite par les glucocorticoïdes est due à des modifications des taux de naissance et de mort des cellules osseuses au moyen d'un modèle murin d'excès de glucocorticoïdes et de biopsies osseuses pratiquées sur des patients atteints d'ostéoporose induite par les glucocorticoïdes. Cette invention démontre que l'administration de glucocorticoïdes accroît l'apoptose des ostéocytes et des ostéoblastes matures et réduit la vitesse de formation osseuse et la densité minérale osseuse associée à des troubles de l'ostéoclastogenèse et de l'ostéoblastogenèse dans la moelle épinière.</p>		

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<b>(54) Title:</b> IN VITRO AND IN VIVO MODELS FOR SCREENING COMPOUNDS TO PREVENT GLUCOCORTICOID-INDUCED BONE DESTRUCTION		
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Description

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5                   **IN VITRO AND IN VIVO MODELS FOR SCREENING**  
**COMPOUNDS TO PREVENT GLUCOCORTICOID-INDUCED BONE**  
                    **DESTRUCTION**

10  
5                   **BACKGROUND OF THE INVENTION**

15                   Cross-Reference to Related Application

                    This application claims benefit of U.S. provisional application  
60/105,805, filed October 27, 1998, now abandoned.

20  
10                  Field of the Invention

25                   The present invention relates generally to bone  
physiology. More specifically, the present invention relates to in  
vitro and in vivo models for screening compounds to prevent  
glucocorticoid-induced bone destruction.

30  
15                  Description of the Related Art

35                   The adverse effects of hypercortisolism on bone have  
been recognized for over 60 years (1), but the precise cellular and  
molecular basis of these changes has remained elusive. Today, the  
40                  iatrogenic form of the disease has become far more common than  
Cushing's syndrome and glucocorticoid-induced osteoporosis is  
now third in frequency following post-menopausal and senile  
osteoporosis (2).

45                   Bone loss due to glucocorticoid excess is diffuse,  
25                  affecting both cortical and cancellous bone, but has a predilection  
for the axial skeleton. Spontaneous fractures of the vertebrae or

5 ribs arc, therefore, often presenting manifestations of the disorder  
(3,4). A cardinal feature of glucocorticoid-induced osteoporosis is  
decreased bone formation (5). In addition, patients receiving  
10 long-term glucocorticoid therapy sometimes develop collapse of  
5 the femoral head (osteonecrosis), but the mechanism underlying  
this is uncertain (6). Decreased bone formation, and *in situ* death  
of isolated segments of the proximal femur suggest that  
15 glucocorticoid excess may alter the birth and death of bone cells.  
Defective osteoblastogenesis has been reported to be linked to  
10 reduced bone formation and age-related osteopenia in the SAMP6  
20 mouse (7). Besides the relationship between aberrant osteoblast  
production and osteoporosis, it has been recently shown that a  
significant proportion of osteoblasts undergo apoptosis (8), which  
25 raises the possibility that the premature or more frequent  
15 occurrence of osteoblast apoptosis could contribute to incomplete  
repair of resorption cavities and loss of bone.

30 Thus, the prior art is deficient in compounds that  
possess the advantageous properties of glucocorticoids, namely  
anti-inflammatory properties, but do not cause bone loss or  
20 osteoporosis. The present invention provides for methods of  
35 screening compounds to fulfill this long-standing need in the art.

## 40 SUMMARY OF THE INVENTION

25 To demonstrate that glucocorticoid-induced bone  
45 disease is due to changes in the birth or death rate of bone cells, a  
murine model of glucocorticoid excess was used as well as bone  
biopsy specimens obtained from patients with glucocorticoid-

5 induced osteoporosis. This invention demonstrates that  
glucocorticoid administration decreases bone formation rate and  
bone mineral density accompanied by defective osteoblastogenesis  
10 and osteoclastogenesis in the bone marrow and increases  
5 apoptosis of mature osteoblasts and osteocytes.

One object of the present invention is to provide  
methods to screen compounds that retain the anti-inflammatory  
15 properties of glucocorticoids yet do not result in bone loss or  
osteoporosis due to apoptosis of osteoblasts and osteocytes.

10 In one embodiment of the present invention, there is  
provided a method of screening for compounds that reduce the  
bone deteriorating effects of glucocorticoids, comprising the steps  
of: (a) contacting osteoblast and osteocyte cells with either a  
25 glucocorticoid alone or a glucocorticoid in combination with a test  
15 compound; and (b) comparing the number of cells undergoing  
apoptosis following treatment with the glucocorticoid alone or  
following treatment with the glucocorticoid in combination with  
30 the test compound; wherein a lower number of apoptotic cells  
following treatment with the glucocorticoid in combination with  
20 the test compound than with the glucocorticoid alone indicates  
that the test compound reduces the bone deteriorating effects of  
the glucocorticoid. This embodiment also includes the  
40 aforementioned method, wherein the compound has little effect on  
the anti-inflammatory properties of the glucocorticoid, further  
25 comprising the step of comparing the anti-inflammatory response  
of the glucocorticoid in combination with the test compound to the  
45 anti-inflammatory response of the glucocorticoid alone; wherein  
essentially equivalent anti-inflammatory responses of the  
glucocorticoid alone and the glucocorticoid in combination with the

5 test compound is indicates that the test compound both reduces  
the bone deteriorating effects, while retaining the anti-  
inflammatory properties of the glucocorticoid; wherein said anti-  
10 inflammatory response is determined by models of inflammation  
5 selected from the group consisting of the adjuvant-induced  
arthritis model and hindlimb inflammation model.

15 In another embodiment of the present invention, there  
is provided a method of screening for glucocorticoid analogs that  
possess decreased apoptotic properties towards osteoblast and  
10 osteocyte cells, comprising the steps of: (a) contacting the cells  
20 with either a glucocorticoid or a glucocorticoid analog; and (b)  
comparing the number of apoptotic cells following treatment with  
the glucocorticoid or the glucocorticoid analog, wherein a lower  
25 number of apoptotic cells following treatment with the  
15 glucocorticoid analog than with the glucocorticoid indicates that  
the glucocorticoid analog possesses decreased apoptotic properties  
towards the cells. This embodiment also includes the  
30 aforementioned method, wherein the glucocorticoid analog retains  
anti-inflammatory properties, further comprising the step of: (c)  
35 20 comparing the anti-inflammatory response of the glucocorticoid in  
combination with a test compound to the anti-inflammatory  
response of the glucocorticoid alone, wherein essentially  
40 equivalent anti-inflammatory responses of the glucocorticoid  
alone and the glucocorticoid in combination with the test  
25 compound is indicative of a glucocorticoid analog that possesses  
decreased apoptotic properties while retaining anti-inflammatory  
45 properties; wherein said anti-inflammatory response is  
determined by models of inflammation selected from the group



5 consisting of the adjuvant-induced arthritis model and hindlimb inflammation model.

10 In yet another embodiment of the present invention, there is provided a method of screening for compounds that  
5 stimulate bone development, comprising the steps of: (a) contacting osteoblast and osteocyte cells with either a glucocorticoid or a test compound; and (b) comparing the number  
15 of cells undergoing apoptosis following treatment with the glucocorticoid or the test compound; wherein a lower number of apoptotic cells following treatment with the test compound than  
20 with the glucocorticoid indicates that the test compound stimulates bone development.

25 In still yet another embodiment of the present invention, there is provided a method of screening for compounds  
15 that increase bone mineral density, comprising the steps of: (a) contacting osteoblast and osteocyte cells with either a glucocorticoid or a test compound; and (b) comparing the number  
30 of cells undergoing apoptosis following treatment with the glucocorticoid and the test compound; wherein a lower number of apoptotic cells following treatment with the test compound than  
35 with the glucocorticoid is indicative of a compound that increases bone mineral density.

40 In the above-mentioned embodiments, contacting is selected from the group consisting of *in vitro* cell cultures and *in*  
25 *vivo* murine animal model and determination of apoptosis is selected from the group consisting of TUNEL, DNA fragmentation and immunohistochemical analysis.

5 Other and further aspects, features, and advantages of  
the present invention will be apparent from the following  
description of the presently preferred embodiments of the  
10 invention. These embodiments are given for the purpose of  
5 disclosure.

## 15 BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features,  
20 advantages and objects of the invention, as well as others which  
will become clear, are attained and can be understood in detail,  
more particular descriptions of the invention briefly summarized  
25 above may be had by reference to certain embodiments thereof  
which are illustrated in the appended drawings. These drawings  
15 form a part of the specification. It is to be noted, however, that  
the appended drawings illustrate preferred embodiments of the  
30 invention and therefore are not to be considered limiting in their  
scope.

35 **Figure 1** shows photomicrographs of the effects of  
20 prednisolone on murine vertebral cancellous bone. In panel A, is  
a longitudinal, panoramic section from a mouse receiving placebo  
and in panel B, a section from a mouse receiving prednisone. The  
40 histomorphometric reading area is outlined. Toluidine blue stain,  
original magnification X25.

25 **Figure 2** shows quantification of CFU-OB and  
45 osteoclast progenitors formed in *ex vivo* bone marrow cell  
cultures. Marrow cells were obtained from the femurs of male  
mice after 27 d of exposure to placebo (white bars) or 2.1  
50

5 mg/kg/d of prednisolone (black bars). Cells from each mouse  
were cultured separately.

10 **Figure 3** shows the effect of prednisolone on murine  
osteoblast apoptosis. Osteoblasts were counted in undecalcified  
5 sections of cancellous bone from the vertebral secondary  
spongiosa. In panel A, the placebo group is shown and in panel B,  
the higher dose prednisolone group. Apoptotic cells in this  
15 experiment were identified using TUNEL and morphometric  
features such as nuclear fragmentation and condensation of  
10 chromatin (arrows). Methyl green counterstain viewed with  
Nomarski differential interference microscopy, original  
20 magnification X400.

25 **Figure 4** shows the effect of prednisolone on murine  
osteocyte apoptosis. The cells were counted in undecalcified  
15 sections of femoral metaphyseal cortical bone. In panel A, the  
placebo group is shown and in panel B, the higher dose  
prednisolone group. Apoptotic osteocytes (arrowheads) are seen  
30 in close proximity to normal cells. Methyl green counterstain  
viewed with Nomarski differential interference microscopy,  
20 original magnification X630.

35 **Figure 5** shows the effect of chronic prednisone  
treatment on apoptosis in human bone. TUNEL-positive  
osteoblasts (arrowheads) and osteocytes (arrows) were absent  
40 from normal subjects (**Figure 5A**) but were clearly identified in  
25 patients with prednisone-induced osteoporosis (**Figure 5B** and  
**Figure 5C**). Approximately 5% of the osteocytes and 30% of the  
45 osteoblasts were apoptotic. The photomicrographs are from  
transiliac bone biopsy specimens. Methyl green counterstain

viewed with Nomarski differential interference microscopy,  
original magnification X630.

## DETAILED DESCRIPTION OF THE INVENTION

5

Glucocorticoid-induced bone disease is characterized  
by decreased bone formation and *in situ* death of isolated  
segments of bone (osteonecrosis) suggesting that glucocorticoid  
excess, the third most common cause of osteoporosis, may affect  
the birth or death rate of bone cells thus reducing their numbers.  
To examine this, prednisolone was administered to 7-month-old  
mice for 27 days and decreased bone density, serum osteocalcin  
and cancellous bone area along with trabecular narrowing were  
found. These changes were accompanied by diminished bone  
formation and turnover, as determined by histomorphometric  
analysis of tetracycline-labeled vertebrae, and impaired  
osteoblastogenesis and osteoclastogenesis, as determined by *ex*  
*vivo* bone marrow cell cultures. In addition, the mice exhibited a  
3-fold increase in osteoblast apoptosis in vertebrae and showed  
apoptosis in 28% of the osteocytes in metaphyseal cortical bone.  
As in mice, an increase in osteoblast and osteocyte apoptosis was  
documented in patients with glucocorticoid-induced osteoporosis.  
Decreased production of osteoclasts explains the reduction in bone  
turnover while decreased production and apoptosis of osteoblasts  
would account for the decline in bone formation and trabecular  
width. Furthermore, accumulation of apoptotic osteocytes may  
contribute to osteonecrosis. These findings provide evidence that  
glucocorticoid-induced bone disease arises from changes in the  
numbers of bone cells.

5           The present invention is directed towards methods of  
screening compounds that retain the anti-inflammatory properties  
of glucocorticoids while lacking the bone degeneration properties  
10           associated with long-term administration due to apoptosis of  
5           osteoblasts and osteocytes.

15           The present invention is further directed towards  
methods of screening compounds that promote bone regeneration  
by inhibiting the apoptosis of osteoblasts and osteocytes.

20           As used herein, the terms "glucocorticoid" and  
10           "glucocorticoid analog" is defined as substances that bind to the  
glucocorticoid receptor.

25           As used herein, the term "apoptosis" refers to  
programmed cell death with nuclear fragmentation and cell  
shrinkage as detected by morphological criteria and Terminal  
15           Uridine Deoxynucleotidyl Transferase Nick End Labeling (TUNEL)  
staining.

30           As used herein, the terms "anti-inflammatory  
response" or "anti-inflammatory property" refers to preventing  
the induction of cytokines and other events that lead to T cell  
35           20           activation. Several models of inflammation are routinely used in  
the art, including the adjuvant-induced arthritis model and  
hindlimb inflammation model which are well known to those  
40           having ordinary skill in this art (54, 55).

45           As used herein, the term "bone mineral density" refers  
25           to bone mass as defined by Dual-Energy X-Ray Absorptiometry  
(DEXA).

5 The following examples are given for the purpose of  
illustrating various embodiments of the invention and are not  
meant to limit the present invention in any fashion:

### 10 EXAMPLE 1

#### Animals

15 Male Swiss Webster mice (Charles River Laboratories,  
Stone Ridge, NY) were electronically tagged (Biomedic Data System  
Inc., Maywood, NJ) and kept in plastic cages (3-5 animals per  
20 cage) under standard laboratory conditions with a 12 hr dark, 12  
hr light cycle and a constant temperature of 20°C and humidity of  
48%. All mice were fed on a standard rodent diet (Agway RMH  
3000, Arlington Heights, IL) containing 22% protein, 5% fat, 5%  
25 fiber, 6% ash, 3.5 Kcal/g, 1.0 IU vitamin D3/g, 0.97% calcium and  
15 0.85% phosphorus with water *ad libitum*. The animals and food  
supply were weighed at one week intervals throughout the  
30 experiment. Studies were approved by the UAMS Division of  
Laboratory and Animal Medicine.

### 35 EXAMPLE 2

#### Glucocorticoid administration--experimental design

40 Bone mineral density (BMD) determinations were done  
at two week intervals to identify the peak adult bone mass of the  
mice, which was reached between 5 and 6 months-of-age (9).  
25 Animals at peak bone mass were used to avoid obscuring the  
negative impact of glucocorticoid excess on bone mineral density  
by the confounding effects of increased linear and radial growth.  
Before the experiment began, bone mineral density measurements

5 were repeated to allocate the animals into groups ( $n = 4 - 5$ ) with  
equivalent spinal density values. The mice (7-mo-old) received  
placebo or prednisolone, a synthetic glucocorticoid analog that  
10 does not require hepatic hydroxylation and has minimal  
5 mineralocorticoid activity, thus eliminating the need for potassium  
supplementation or sodium restriction (10,11). Implantation of  
pellets releasing 0.5 mg/kg/d of prednisolone (the no effect dose)  
15 did not decrease bone mineral density. Therefore, two doses were  
used, 0.7 mg/kg/d (lower dose) and 2.1 mg/kg/d (higher dose),  
10 chosen from pilot studies to bracket the dose (1.4 mg/kg/d) that  
invariably causes densitometric evidence of bone loss. These  
20 doses were administered for 27 days by subcutaneous  
implantation of slow-release pellets (Innovative Research of  
America, Sarasota, FL). Bone mineral density measurements were  
25 obtained at the beginning of the experiment and 27 days post-  
implantation. For dynamic histomorphometric measurements,  
tetracycline HCl (30 mg/kg body weight) was given  
30 intraperitoneally 17 and 23 days post-implantation. After 27  
days, the mice were sacrificed, serum and urine specimens were  
20 taken, bone marrow aspirates were obtained from the right femur  
for *ex vivo* marrow cell cultures and the left femur and lumbar  
35 vertebrae were prepared for histomorphometric analysis. Livers  
were examined for fatty infiltration as a sign of prednisolone  
40 toxicity. The weight of the seminal vesicles (mg/100 g body  
25 weight) was used as an index of the androgen status of the  
animals (12). To help interpret these measurements, a separate  
45 group of animals was orchidectomized ( $n = 5$ ).

EXAMPLE 3Bone densitometry

Dual-energy X-ray absorptiometry (DEXA) was used to determine global (whole body minus the head), spinal and hindquarters bone mineral density in live mice (7,9). The scans done at 27 days after pellet implantation were analyzed using the 'Compare' technique, in which the evaluation is based on the exact positioning and region of interest placement of the baseline scan. Accuracy of the DEXA measurements was demonstrated by the strong linear relationship between ash weight and bone mineral content at each region (7). Over the 18 months, the coefficient of variation for the bone mineral density of a plastic-embedded whole mouse skeleton was 3.0% (n = 146).

EXAMPLE 4Serum and urine biochemical measurements

Serum osteocalcin was measured by radioimmunoassay using a goat anti-murine osteocalcin and murine osteocalcin as tracer and standard (Biomedical Technologies, Stoughton, MA). Urinary free deoxypyridinoline excretion was determined by a microtiter competitive enzyme immunoassay (Pyrilinks-D, Metra Biosystems, Mountain View, CA) and was expressed as a ratio to the urinary creatinine.

EXAMPLE 5Bone histomorphometric analysis

The distal femora and lumbar vertebrae were fixed in 4°C Millonig's phosphate-buffered 10% formalin, pH 7.4, embedded undecalcified in methyl methacrylate and stained



(7,9,13). The histomorphometric examination was done with a computer and digitizer tablet (OsteoMetrics Inc. Version 3.00, Atlanta, GA) interfaced to a Zeiss Axioscope (Carl Zeiss, Inc., Thornwood, NY) with a drawing tube attachment. All cancellous measurements were two-dimensional, confined to the secondary spongiosa and made at X400 magnification (numerical aperture 0.75). The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (14). The trabecular width and osteoid width were measured directly. Trabecular spacing and number were calculated (15). Only TRAPase-positive cells were included in the osteoclast perimeter. The rate of bone formation ( $\mu\text{m}^2/\mu\text{m}/\text{d}$ ) and turnover ( $\%/ \text{d}$ ) were calculated (7).

#### EXAMPLE 6

##### Detection and quantification of osteoblasts and osteoclasts in *ex vivo* bone marrow cultures

One femur from each mouse was flushed with 5 ml of phenol red-free  $\alpha$ MEM (Gibco BRL, Gaithersburg, MD) containing 10% FBS (Hyclone, Logan, UT) to obtain marrow cells. After the cells were rinsed and resuspended to obtain a single cell suspension, the nucleated cell count was determined using a Coulter Counter. Cells from each animal were cultured separately.

The number of colony-forming unit-fibroblast (CFU-F) and CFU-osteoblast (CFU-OB) present in the bone marrow preparations were determined (16-18). Briefly, cells were seeded at  $1.5 \times 10^6$  per  $10 \text{ cm}^2$  well for the determination of CFU-F number and maintained for 10 days in phenol red-free  $\alpha$ MEM

5 containing 15% preselected FBS, 50  $\mu$ M ascorbic acid and 10 mM  $\beta$ -  
glycerophosphate (Sigma Chemical Co, St. Louis, MO) with one-half  
of the medium replaced after 5 days. After fixation in neutral  
10 buffered formalin and staining with hematoxylin, colonies  
5 containing a minimum of 20 fibroblastoid cells were enumerated.  
Cells were seeded at  $2.5 \times 10^6$  cells per 10  $\text{cm}^2$  well for the  
determination of CFU-OB number and cultured for 25-28 days as  
15 described above for CFU-F. After fixation in 50% ethanol and 18%  
formaldehyde, cultures were stained using Von Kossa's method to  
10 visualize and enumerate colonies containing mineralized bone  
20 matrix.

Osteoclast formation in bone marrow cultures was  
assessed in replicate cultures (4-6 from each animal) maintained  
25 for 9 days in the presence of  $\alpha$ MEM, 10% FBS and 10 nM  
15  $1.25(\text{OH})_2\text{D}_3$  (7). Briefly, marrow cells were cultured at  $1.5 \times 10^6$   
per 2  $\text{cm}^2$  well on 13 mm round Thermanox disks and maintained  
for 8 days in the presence of 10% FBS in  $\alpha$ MEM supplemented  
30 with  $10^{-8}$  M  $1.25(\text{OH})_2\text{D}_3$  (provided by Dr. Milan Uskokovic,  
Hoffman-LaRoche, Nutley, NJ). At the end of the experiment, cells  
35 20 were processed for the autoradiographic detection of bound  $^{125}\text{I}$ -  
calcitonin ( $^{125}\text{I}$ -CT) and stained for tartrate-resistant acid  
phosphatase. Because many osteoclasts in murine bone possess  
40 only one nucleus (7), it is impossible to distinguish between  
preosteoclasts and mononuclear osteoclasts in *ex vivo* cultures of  
25 murine bone marrow cells. Therefore, mononucleated and  
multinucleated cells that both bind  $^{125}\text{I}$ -CT and express TRAPase  
45 were designated as osteoclastic cells. The number of osteoclasts  
formed in this assay is a reflection of the number of osteoclast  
progenitors present in the bone marrow aspirate and the number

of stromal/osteoblastic support cells that form during the culture period.

The number of CFU-F colonies, CFU-OB colonies, and osteoclastic cells formed from the marrow cells of each animal was expressed as the number per femur, which was calculated by multiplying the number of colonies or osteoclasts obtained per  $10^6$  cells seeded at the initiation of the cultures by the total number of marrow cells obtained from the animal.

#### EXAMPLE 7

##### Measurement of apoptosis in undecalcified bone sections

Sections were mounted on silane-coated glass slides (Scientific Device Lab, Inc., Des Plains, IL), deplasticized and incubated in 10 mM citrate buffer, pH 7.6, in a microwave oven at 98°C for 5 minutes. Slides were then incubated with 0.5% pepsin for 30 minutes at 37°C. Apoptotic cells were detected by the TUNEL reaction (transferase-mediated biotin-dUTP nick end-labeling) using Klenow terminal deoxynucleotidyl transferase (Oncor, Gaithersburg, MD) in sections counterstained with 1% methyl green. The TUNEL reaction was noted within cell nuclei and the cells whose nuclei were clearly brown from the peroxidase-labeled anti-digoxigenin antibody instead of the blue-green from the methyl green were interpreted as positive. Plastic-embedded sections of weaned rat mammary tissue were used as a positive control. Negative controls were made by omitting the transferase. Morphological changes characteristic of apoptosis were examined carefully to minimize ambiguity regarding the interpretation of results. With these precautions,

5 TUNEL has been unequivocally associated with apoptosis (19). In  
addition, TUNEL has been used with DNA fragmentation and  
immunohistochemical studies to demonstrate apoptosis of  
10 osteoblastic cells and osteoblasts both *in vitro* and *in vivo* (8,20).  
5 Apoptosis was also assessed in transiliac bone biopsy specimens  
taken from two patients with glucocorticoid-induced osteoporosis  
(22- and 36-yr-old, receiving 15 to 25 mg/d of prednisone for 3  
15 to 6 yr) and from 12 age-, sex- and race-matched controls (13).  
Two longitudinal sections were examined from each patient and  
10 control subject. Osteoblasts were identified as cuboidal cells lining  
the osteoid-covered trabecular perimeter (7,9,13). Osteocytes  
20 were identified inside lacunae in mineralized bone.

#### 25 EXAMPLE 8

##### 15 Statistics

30 Differences in the bone densitometry values were  
determined using the percentage change in BMD from baseline.  
Dose response relations were tested by one-way ANOVA. To  
further evaluate changes in bone histomorphometry, a Student's t  
35 20 test was used to assess for significant differences between group  
means, after testing for equivalence of variances and normal  
distribution of data. The significance of the relative frequency of  
40 apoptotic cells was determined with the  $\chi^2$  statistic. P values less  
than 0.05 were considered significant (21).

#### 25 EXAMPLE 9

##### 45 Demonstration of bone loss in mice receiving prednisolone

In mice implanted with the higher dose of  
prednisolone, global and spinal BMD at 27 days were significantly  
50

5 lower than those found in the mice that were implanted with  
placebo pellets (TABLE I). The decrease in global bone mineral  
density was dose dependent ( $P < 0.05$ ). Demonstrating the  
10 expected propensity for the axial skeleton, glucocorticoid-induced  
5 loss of bone mineral density was less conspicuous at the  
hindquarters. The levels of serum osteocalcin, a marker of  
osteoblast activity, were decreased more than 50% when  
15 compared to placebo, while urinary deoxypyridinoline excretion  
was not significantly different between the groups (TABLE I).  
10 These effects were not due to changes in food intake, body weight  
or androgen status (TABLE II). In addition, hepatic fatty  
20 infiltration was absent.

TABLE I

Bone Mineral Density (BMD) and Serum and Urine Biochemical  
Measurements in Prednisolone-treated Mice

Measurement	Placebo	0.7 mg/kg/d	2.1 mg/kg/d
Global BMD (% change)	-2.7 ± 2.1	-5.0 ± 2.2*	-6.6 ± 1.9†
Spinal BMD (% change)	-3.1 ± 3.0	-6.8 ± 3.2	-8.7 ± 3.5*
Hindquarters BMD (% change)	0.4 ± 10.4	-3.8 ± 8.0	-3.4 ± 6.9
Osteocalcin (µg/L)	93.8 ± 11.5	63.0 ± 27.7*	46.4 ± 13.8†
Deoxypyridinoline (µM/mM creatinine)	78.3 ± 9.3	63.6 ± 14.7	81.5 ± 11.3

Data shown are the mean ± SD from 5-7 animals. \*P < 0.05 vs placebo; †P < 0.005 vs placebo.

TABLE II

Food Intake, Body Weight and Seminal Vesicle Weight in  
Prednisolone-treated Mice

Measurement	Placebo	0.7 mg/kg/d	2.1 mg/kg/d
Food Intake (g/d)	3.4 ± 0.6	3.6 ± 0.2	3.7 ± 0.4
Body Weight (g)	37.9 ± 6.0	33.8 ± 4.3	32.2 ± 4.2
Seminal Vesicle Weight (mg/100 g body weight)	74.6 ± 14.6	92.7 ± 8.7	83.1 ± 6.9

Data=mean ± SD. Seminal vesicle weight in a orchidectomized control was 11.3 ± 3.1 mg/100 g body weight, P <0.001 vs treated mice.)

EXAMPLE 10

Effects of glucocorticoid administration on vertebral bone  
histomorphometry

Consistent with the bone mineral density results, in the animals receiving the higher dose, there was a 40% decline in the vertebral cancellous bone area and a 23% decline in trabecular width (P <0.01) (TABLE III). In both prednisolone groups, there was a trend towards increased trabecular spacing and there was decreased trabecular number in the lower dose group indicating that some trabecular profiles were entirely resorbed.

In the higher dose group, osteoid area decreased by 29%, osteoid perimeter by 34% and osteoid width by 27% (P <0.01). A trend toward decreased osteoblast and osteoclast perimeters was found in the animals receiving the higher dose.

5 There was, however, a 3-fold increase in the empty erosion  
cavities (devoid of osteoclasts) or reversal perimeter. The  
tetracycline-based histomorphometry showed that prednisolone  
10 administration caused a 26% decrease in the mineralizing  
5 perimeter ( $P < 0.05$ ). In addition, a dose-dependent decrease in  
the mineral appositional rate was noted ( $P < 0.05$ ); this decline was  
22% with the lower dose and 40% with the higher dose.  
15 Furthermore, there was a 53% decrease in the rate of bone  
formation with the higher dose ( $P < 0.01$ ), which correlated with  
10 the vertebral cancellous bone area ( $r = 0.57$ ,  $P < 0.05$ ), indicating  
20 that the glucocorticoid-induced decreases in bone area were  
associated with a reduction in the rate of bone formation. Bone  
turnover, expressed as a percentage of the bone area per day, also  
25 decreased in a dose-dependent manner ( $P < 0.05$ ).



TABLE III

Vertebral Cancellous Bone Histomorphometry in Swiss Webster  
Mice After 27 Days of Prednisolone Administration

## Histomorphometric

<u>Determination</u>	<u>Placebo</u>	<u>0.7 mg/kg/d</u>	<u>2.1 mg/kg/d</u>
Bone area/Tissue area(%)	10.4 ± 1.4	6.9 ± 2.1	6.3 ± 1.7†
Trabecular width (µm)	48.0 ± 2.4	48.6 ± 4.3	37.1 ± 4.4†
Trabecular spacing (µm)	423 ± 69	712 ± 302	546 ± 125
Trabecular number (per mm)	1.66 ± 0.6	1.44 ± 0.47	1.77 ± 0.33
Osteoid area/Bone area (%)	2.1 ± 0.2	2.2 ± 0.8	1.5 ± 0.2†
Osteoid perimeter/Bone perimeter			
(%)	15.1 ± 2.1	15.8 ± 5.1	9.9 ± 1.1†
Osteoid width (µm)	2.6 ± 0.4	2.0 ± 0.3	1.9 ± 0.3*
Osteoblast perimeter/Bone perimeter			
(%)	1.2 ± 0.9	2.2 ± 0.2	0.5 ± 0.4
Osteoclast perimeter/Bone perimeter			
(%)	2.7 ± 1.1	2.6 ± 0.5	1.1 ± 1.7
Reversal perimeter/Bone perimeter			
	2.5 ± 2.3	3.2 ± 2.2	7.2 ± 1.1†
Mineralizing perimeter/Bone perimeter			
(%)	12.9 ± 0.5	13.9 ± 5.6	9.5 ± 2.5*
Mineral appositional rate			
(µm/d)	1.23 ± 0.11	0.96 ± 0.11*	0.74 ± 0.20†
Bone formation rate/Bone perimeter			
(µm <sup>2</sup> /(m/d)	0.15 ± 0.02	0.13 ± 0.04	0.07 ± 0.03†

Bone turnover (%/d)  $0.68 \pm 0.09$   $0.46 \pm 0.12^*$   $0.24 \pm 0.11^\dagger$

Data shown are the mean  $\pm$  SD. There are 4-5 animals per group. \*P < 0.05 vs. placebo;  $\dagger$ P < 0.01 vs. placebo.

### EXAMPLE 11

#### Effects of glucocorticoid administration on osteoblastogenesis and osteoclastogenesis

In bone marrow cell cultures from the animals receiving the higher dose, there was no significant change in CFU-F colonies ( $1250 \pm 374$  vs.  $698 \pm 104$ , NS). However, the number of CFU-OB colonies decreased by 86% ( $375 \pm 257$  SD vs.  $54 \pm 14$ , P < 0.05) and the number of osteoclastic cells formed in response to  $1.25(\text{OH})_2\text{D}_3$  in *ex vivo* marrow cultures decreased by 65% ( $1387 \pm 920$  vs.  $492 \pm 311$ , P < 0.05) (Figure 2).

### EXAMPLE 12

#### Effects of glucocorticoid administration on apoptosis

Counting a total of 973 osteoblasts, there was a 3-fold increase in osteoblast apoptosis in the vertebral cancellous bone of mice receiving the higher dose of prednisolone when compared to controls ( $2.03\% \pm 0.34$  vs.  $0.66\% \pm 0.07$ , P < 0.05). Morphological changes typical of apoptosis accompanied the TUNEL-positive osteoblasts and included sharply defined, condensed chromatin plastered against the nuclear membrane, nuclear fragmentation and cell shrinkage (Figure 3A and 3B).

5 In addition, prednisolone caused the appearance of  
apoptotic osteocytes in cortical bone sections taken from femora  
(Figure 4A and 4B). Whereas none of the osteocytes exhibited  
10 apoptotic features in the control animals, 28% of 131 cortical  
5 osteocytes were apoptotic in the animals receiving the higher  
dose. Osteocyte apoptosis was restricted to small groups of cells in  
the center of the femoral metaphyseal cortex and were absent  
15 from vertebral cortical bone. The apoptotic osteocytes were  
identified in close proximity to normal osteocytes, in contrast to  
10 the large homogenous areas of dead and dying cells typical of cell  
20 necrosis. An increase in apoptotic hypertrophic chondrocytes and  
bone marrow cells was also noted in mice receiving either dose of  
prednisolone. Osteoclast apoptosis was not observed.

### 15 EXAMPLE 13

#### 30 Demonstration of apoptotic osteoblasts and osteocytes in patients with glucocorticoid-induced osteoporosis

In transiliac bone biopsies taken from two patients,  
TUNEL-positive osteoblasts and osteocytes were clearly identified  
35 20 in both (Figure 5B and 5C) but were absent from specimens taken  
from 12 age-, sex- and race-matched controls (Figure 5A). As in  
the murine model, bone histomorphometry from these two  
40 patients showed the changes expected with chronic glucocorticoid  
therapy (5): reduced cancellous bone area (11.1 and 8.8%, normal  
25 is  $22.4 \pm 1.2$  SEM), decreased trabecular width (62 and 118  $\mu\text{m}$ ,  
normal is  $161 \pm 9$ ), decreased osteoblast perimeter (2.1 and 2.3%,  
45 normal is  $7.6 \pm 0.4$ ), decreased osteoclast perimeter (0 and 0.4%,  
normal is  $0.9 \pm 0.2$ ), increased reversal perimeter (13.5 and 15.4%,

5 normal is  $6.9 \pm 0.7$ ) and diminished bone formation rate ( $0.02$  and  
0.05  $\mu\text{m}^2/\mu\text{m}/\text{d}$ , normal is  $0.095 \pm 0.012$ ). In the cancellous bone  
10 of these specimens, approximately 5% of the osteocytes and 30% of  
the osteoblasts were apoptotic. Apoptosis of osteoclasts or cortical  
5 osteocytes was not observed. A transiliac bone biopsy represents  
a much smaller sample of the human skeleton than the murine  
15 femur and lumbar vertebrae represent of the mouse skeleton.  
Therefore, it is not surprising that the percentage of apoptotic  
osteoblasts and osteoclasts was different in the human and murine  
20 specimens.

#### EXAMPLE 14

##### Early effects on bone resorption

To directly establish whether glucocorticoids initially  
15 accelerate bone resorption in the mouse, the vertebral cancellous  
bone histology were examined in an additional group of somewhat  
30 younger mice (5-mo-old) after 7 days administration of the higher  
dose of prednisolone or placebo ( $n = 5$ ). It was found that  
whereas prednisolone caused a 59% decrease in the osteoblast  
35 perimeter ( $5.2\% \pm 1.5$  SD vs.  $2.1 \pm 1.1$ ,  $P < 0.005$ ), the osteoclast  
20 perimeter increased 96% ( $0.51\% \pm 0.34$  vs.  $1.00 \pm 0.41$ ,  $P < 0.05$ ).

##### Summary

The choice of the mouse for these studies was based on  
45 its validity as a model of the bone loss associated with loss of sex  
steroids (9,22) and with senescence (7), but the mouse also has  
several advantages over other animals (TABLE IV). In the mouse,

5 glucocorticoid administration consistently induces axial, greater  
than appendicular, bone loss without weight loss or  
hypogonadism, accompanied by histological indices of impaired  
10 osteoblast function, thus reproducing the major features of the  
5 human disease (2-5). Although the doses used in the studies  
described herein were higher in relation to body weight than in  
humans, they were only mildly higher than the dose determined  
15 by serial bone densitometry to have no effect and were consistent  
with the much higher metabolic clearance of glucocorticoids and  
10 other compounds in laboratory animals than in humans (35-37).  
20 Nonetheless, the similarity of the glucocorticoid-induced increases  
in apoptotic cells and bone histomorphometric features in mice  
and humans indicates that the observations in the mouse are not  
25 due to pharmacological differences.

15 The effects of glucocorticoids were examined after 27  
days, a period equivalent in the mouse to about 3 to 4 years in  
humans. Thus, these findings represent long-term, rather than  
30 acute effects. Although a significant correlation was found  
between the severity of the cancellous bone loss and the extent of  
20 reduction in bone formation, several other lines of evidence imply  
35 that some of the observed bone loss was due to an early increase  
in bone resorption which had subsided by the time of  
examination. First, there was suggestive evidence of complete loss  
40 of some trabeculae (TABLE III). Second, based on the bone  
25 turnover measured in the placebo group which must be close to  
the rate found in all the animals at the beginning of the study,  
45 even with total suppression of bone formation, the initial rate of  
bone turnover could have accounted only for an exponential  
decline in cancellous bone area of 18%, whereas a 40% decrease

5 was observed. Finally, an early increase in osteoclast perimeter was confirmed by histomorphometric examination of vertebral cancellous bone after 7 days of prednisolone administration.

10 By 27 days of prednisolone administration, bone resorption fell to, or below, normal, as indicated by the downward trend in the osteoclast perimeter, normal urinary deoxypyridinoline excretion and profound decrease in  
15 osteoclastogenesis. The persistent increase in erosion cavities devoid of osteoclasts, measured as the reversal perimeter, merely  
10 indicates delayed bone formation (38), and has been previously observed in glucocorticoid-treated patients (5,39). Consequently,  
20 the present invention emphasizes the relevant findings at 27 days to chronic, rather than short-term, glucocorticoid administration to humans.

25 15 Vertebral cancellous bone in adult mice undergoes sequential, coupled bone remodeling that is qualitatively similar to that occurring in human bone (7,9). Many of the changes in cellular, osteoid and tetracycline-based histological indices induced by glucocorticoid administration can be accounted for by  
30 a reduction in the activation frequency of bone remodeling, the main determinant of the rate of bone turnover (40), which is an inevitable consequence of the substantial decrease in osteoclastogenesis that was observed. Although a reduction in  
35 bone turnover will not by itself cause bone loss, the decrease in trabecular width, which was the major structural change  
40 25 observed, is usually the result of incomplete cavity repair. This is, at least in part, due to inadequate osteoblast recruitment, either from diminished production or ineffective migration to the bone surface (40). The reduction in osteoblastogenesis was of sufficient

5 magnitude to explain the decrease in bone formation rate, and  
would also have contributed to the inadequate osteoblast  
recruitment and consequent decline in trabecular width. Thus, the  
10 inhibitory effect of glucocorticoids on early bone cell progenitors  
5 in the bone marrow can account for many of the *in vivo*  
observations.

15 The data herein also bear on recent ideas concerning  
the relationships between early osteoblast and osteoclast  
progenitors in the bone marrow. Although mature osteoclasts and  
10 osteoblasts are needed successively at each bone surface site that  
is being remodeled, these cells are needed simultaneously as the  
20 basic multicellular unit (which is the instrument of bone  
remodeling) progresses through or across the surface of bone (41).  
The necessary parallel production of executive cells is  
25 accomplished by signals that originate from early members of the  
stromal cell-osteoblast family, which support in various ways the  
30 production of mononuclear preosteoclasts in the bone marrow  
(42). The demonstration herein of a marked reduction in the  
numbers of both CFU-OB and osteoclast progenitors derived from  
20 *ex vivo* bone marrow cell cultures makes it likely that  
glucocorticoid administration inhibits the proliferation and/or  
35 differentiation of the stromal cell-osteoblast family at an early  
stage, leading to a reduction in the number of mature, matrix-  
40 secreting osteoblasts as well as the osteoblastic cells that support  
osteoclast development. A direct inhibitory effect of  
25 glucocorticoids on osteoclast precursor proliferation is not  
excluded by the data herein, but would be less easy to reconcile  
45 with the finding of an early increase in the osteoclast perimeter.

5                   Some osteoblasts become osteocytes and some become  
lining cells, but these fates combined do not account for all the  
osteoblasts initially present. Although migration along or away  
10                   5   most likely alternative fate (43). Osteoblasts in remodeling bone  
undergo apoptosis with a frequency sufficient to account for most  
or all of those missing (8). Based on the dynamic  
15                   histomorphometry at the murine vertebral secondary spongiosa  
and a wall width of about 15  $\mu\text{m}$  (7,9,14), the mean active life  
20                   10 span of an osteoblast was calculated on cancellous bone by  
dividing wall width by the mineral appositional rate. From this  
calculation, the mean active lifespan of a murine osteoblast is  
25                   about 12 days or 288 hours. The prevalence of osteoblast  
apoptosis in the present study was 0.0066 in the placebo group.  
15                   The following relationship was applied:

$$t_{Ap}/288 = 0.0066/f_{Ap},$$

30                   where  $t_{Ap}$  is the mean duration (in hours) of the DNA  
fragmentation phase of apoptosis that is detected by TUNEL, and  
 $f_{Ap}$  is the fraction of osteoblasts that undergoes apoptosis and  
35                   20 based on a value of  $t_{Ap}$  of about 3 hours, determined previously  
for regenerating liver (44), the corresponding value for  $f_{Ap}$  in the  
placebo group is 0.6. Thus, the low prevalence of apoptosis in the  
40                   placebo group is consistent with studies of human bone that 50-  
70% of osteoblasts undergo apoptosis, and that only a minority  
25                   become osteocytes or lining cells (43).

45                   In the animals receiving the higher dose of  
prednisolone, the prevalence of apoptosis was 0.0203. With  
prednisolone administration, phagocytosis of the apoptotic cells  
would be suppressed and it was estimated that  $t_{Ap}$  could be



5 doubled (45). Wall width was reduced to about 8  $\mu\text{m}$  and mineral  
appositional rate to 0.74  $\mu\text{m}/\text{d}$ , so that the active lifespan of an  
osteoblast is about 260 hours. In these circumstances, the  
10 corresponding value for  $f_{\text{Ap}}$  in the prednisolone group is 0.9.

5 Although there is some uncertainty to the assumptions used for  
these estimates, the approach does help explain the data and  
disclose the devastating impact of glucocorticoid excess on  
15 osteoblast survival. The higher proportion of osteoblasts showing  
features of apoptosis in glucocorticoid-treated mice and human  
10 subjects could indicate no more than prolongation of the time  
needed for completion of the process, but it is more likely that  
glucocorticoids induce apoptosis, either prematurely in cells  
already destined for this fate or in cells otherwise destined to  
25 become lining cells or osteocytes. In either case, the mean active  
15 lifespan of osteoblasts would be shortened and less bone formed.  
Thus, the reduction in bone formation by glucocorticoids could be  
due to increased death as well as decreased birth of osteoblasts.

Osteocytes are long-lived but not immortal cells. In  
human rib cortical bone, their lifespan has been estimated at  
35 20 about 20 years (47); if bone remains unremodeled for a longer  
time, the osteocytes die, as revealed by empty lacunae and  
hypermineralized perilacunar bone, referred to as micropetrosis  
40 (48). Osteocyte death in cancellous bone, indicated by absence of  
lactic dehydrogenase activity, increases in prevalence with age in  
25 the upper femur but not in the vertebrae (49), probably because  
of the higher bone turnover in the spine. Empty lacunae and  
enzyme absence can reveal the fact, but not the mode, of death.  
45 Osteocyte apoptosis has recently been detected in human iliac  
cancellous bone and its prevalence was increased by

5 pharmacological induction of estrogen deficiency (19). The  
present invention demonstrated that chronic glucocorticoid  
administration, both to mice and to human patients, likewise  
10 increases the prevalence of osteocyte apoptosis. The proportion of  
5 apoptotic osteocytes was much higher than of osteoblasts,  
reflecting the unique unavailability of osteocytes for phagocytosis  
because of their anatomic isolation from scavenger cells, and the  
15 need for extensive degradation to small molecules to dispose of  
the cells through the narrow canaliculi. As a result, the process is  
10 prolonged and affected cells accumulate.

20 The network of osteocytes probably participates in the  
detection of microdamage and the transmission of signals that lead  
to its repair by remodeling (50). Disruption of the network by  
osteocyte apoptosis could compromise this mechanism, leading to  
25 microdamage accumulation and increased bone fragility (51).  
Second, chronic glucocorticoid administration sometimes leads to  
so-called aseptic or avascular necrosis of bone (6). Glucocorticoid-  
induced osteocyte apoptosis, a cumulative and unrepairable  
30 defect, would explain the correlation between total dose and  
incidence of avascular necrosis of bone (53) and its occurrence  
20 after glucocorticoid administration had ceased.

35 In conclusion, the present invention has demonstrated  
that the mouse is a valid and informative model of glucocorticoid-  
induced bone disease, not confounded by weight loss or sex-  
40 steroid deficiency, and that many of the effects of chronic  
25 glucocorticoid administration on bone can be explained by  
decreased birth of osteoblast and osteoclast precursors and  
45 increased apoptosis of mature osteoblasts and osteocytes.

TABLE IV

Confounding Factors with Glucocorticoid AdministrationAnimalsFactors

5	Rats (23,24)	Paradoxical increase in cancellous bone mass*, decreased food intake and weight.
15	Rabbits and dogs (25-27)	Inconsistent changes in bone density and cancellous bone area, weight loss, hepatic fatty infiltration.
10	Ewes (28-30)	Histological changes resemble glucocorticoid-treated patients but corresponding changes in bone density and cancellous bone area are inconsistent.

\*Glucocorticoids inhibit bone resorption and promote apoptosis in rat osteoclasts *in vitro* (31), whereas bone resorption is stimulated in neonatal mouse calvaria (32). Glucocorticoids stimulate bone nodule formation from rat calvarial cells *in vitro* (33) but inhibit differentiation in a murine osteoblastic cell line (34).

The following references were cited herein:

1. Cushing, H. 1932. Bull. Johns Hopkins Hosp. 50:137-195.
2. Lukert, B. 1996. Glucocorticoid-induced osteoporosis. In Osteoporosis. R. Marcus, D. Feldman, and J. Kelsey, editors. Academic Press, San Diego, CA. 801-820.
3. Fitzpatrick, L.A. 1994. Glucocorticoid-induced osteoporosis. In Osteoporosis. R. Marcus, editor. Blackwell Scientific Publications, Boston, MA. 202-226.
4. Reid, I.R. 1989. Clin. Endocrinol. 30:83-103.
5. Dempster, D. 1989. J. Bone Miner. Res. 4:137-141.
6. Mankin, H.J. 1992. N. Engl. J. Med. 326:1473-1479.

- 5 7. Jilka, R.L., et al. 1996. J. Clin. Invest. 97:1732-1740.
8. Jilka, R.L., et al. 1998. J. Bone Miner. Res. 13: (in press).
9. Weinstein, R.S., et al. 1997. Endocrinol. 138: 4013-4021.
- 10 10. Frey, F.J. 1987. Endo. Rev. 8:453-473.
- 5 11. Cope, C.L. 1972. The synthetic analogues. In Adrenal steroids and disease. Lippincott, Philadelphia, PA. 488-491.
12. Broulik, P.D., and L. Starka. 1997. Bone 20:473-475.
- 15 13. Weinstein, R.S., and N.H. Bell. 1988. N. Engl. J. Med. 319:1698-1701.
- 10 14. Parfitt, A.M., et al. 1987. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J. Bone Miner. Res. 2:595-610.
- 20 15. Parfitt, A.M., et al. 1983. J. Clin. Invest. 72:1396-1409.
- 25 16. Bellows, C.G., et al. 1991. Bone Miner. 14:27-40.
17. Owen, M. 1985. Lineage of osteogenic cells and their relationship to the stromal system In W.A. Peck, editor. Bone and Mineral Research. Elsevier, Amsterdam, vol 3:1-25.
- 30 18. Falla, N., et al. 1993. Blood 82:3580-3591.
- 20 19. Tomkinson, A., et al. 1997. J. Clin. Endocrinol. Metab. 82:3128-3135.
- 35 20. Lynch, M.P., et al. 1998. J Cell Biochem 68: 31-49.
21. StatCorp. 1995. Stata Statistical Software Release 4.0. Stata Corporation, College Station, TX, 1-1601.
- 40 22. Jilka, R.L., et al. 1992. Science 257:88-91.
23. King, C.S., et al. 1996. Calcif. Tissue Int. 59: 184-191.
- 45 24. Li, M., et al. 1996. Bone 19:81-88.
25. Quarles, L.D. 1992. Am. J. Physiol. (Endocrinol. Metab.) 263:E136-E141.

- 5 26. Grardel, B., et al. 1994. Osteoporosis Int. 4:204-210.
27. Kawai, K., et al. 1985. J. Bone Joint Surg. 67A:755-762.
28. Deloffre, P., et al. 1995. Bone 17:409S-414S.
- 10 29. Chavassieux, P., et al. 1997. Bone 20:451-455.
- 5 30. Newman, E., et al. 1995. Bone 16:277S-284S.
31. Dempster, D.W., et al. 1997. J. Endocrinol. 154:397-406.
32. Conaway, H, et al. 1996. J. Bone Miner. Metab. 11:1419-1429.
- 15 33. Bellows, C.G., et al. 1987. Endocrinol. 121:1985-1992.
34. Lian, J.B., et al. 1997. Endocrinol. 138:2117-2127.
- 10 35. Stanton, B., et al. 1985. J. Clin. Invest. 75:1317-1326.
- 20 36. Borchard, R.E., et al. 1992. Drug dosage in laboratory animals:  
a handbook. CRC Press, Inc., Boca Raton, FL. 514-517.
37. Kleiber, M. 1961. The fire of life: an introduction to animal  
25 energetics. John Wiley & Sons, Inc. New York. Chapters 10:177-  
15 216 and 11:217-230.
38. Klein, M., et al. 1965. Acta Orthop. Scandinav. 35:171-184.
- 30 39. Bressot, C., et al. 1979. Metab. Bone Dis. & Rel. Res. 1:303-  
311.
40. Parfitt, A.M., et al. 1995. J. Bone Miner. Res. 10:466-473.
- 20 41. Parfitt, A.M. 1994. J. Cell. Biochem. 55:273-286.
- 35 42. Manolagas, S.C., et al. Interleukin-6-type cytokines and their  
receptors. 1996. In Principles of Bone Biology. J.Bilezikian, et al.,  
editors. Academic Press, San Diego, CA. 701-713
- 40 43. Parfitt, A.M. 1990. Bone-forming cells in clinical conditions.
- 25 In Bone: A Treatise, Vol. 1. The Osteoblast and Osteocyte. B.K. Hall,  
editor. Telford and CRC Press, Boca Raton, FL. 351-429.
- 45 44. Bursch, W., et al. 1990. Carcinogenesis. 11:847-583.
45. Cline, M.J. 1974. N. Engl. J. Med. 291:1187-1188.
46. Gohel, et al., 1997. J. Bone Miner. Res. 12 (1):S284. (Abstr.)

- 5 47. Frost, H.M.. 1960. J. Bone Joint Surg. 42A:138-143.  
48. Frost, H.M. 1960. J. Bone Joint Surg. 42A:144-150.  
49. Dunstan, C.R., et al. 1993. Calcif. Tissue Int. 53:S113-117.  
10 50. Aarden, E.M., et al. 1994. J. Cell. Biochem. 55:287-299.  
5 51. Noble, B.S., et al. 1997. Bone 20:273-282.  
52. Ficat, R.P. 1985. J. Bone Joint Surg. 67B:3-9.  
53. Felson, D.T., and J.J. Anderson. 1987. Lancet I:902-905.  
15 54. Knight, B. et al. 1992. Clin. Exp. Immunol. 90:459-465.  
55. Henriques, et al. 1987. Braz. J. Med. Biol. Res. 20: 243-249.

10 Any patents or publications mentioned in this  
20 specification are indicative of the levels of those skilled in the art  
to which the invention pertains. Further, these patents and  
publications are incorporated by reference herein to the same  
25 extent as if each individual publication was specifically and  
15 individually indicated to be incorporated by reference.

30 One skilled in the art will appreciate readily that the  
present invention is well adapted to carry out the objects and  
obtain the ends and advantages mentioned, as well as those  
objects, ends and advantages inherent herein. The present  
35 20 examples, along with the methods, procedures, treatments,  
molecules, and specific compounds described herein are presently  
representative of preferred embodiments, are exemplary, and are  
not intended as limitations on the scope of the invention. Changes  
40 therein and other uses will occur to those skilled in the art which  
25 are encompassed within the spirit of the invention as defined by  
the scope of the claims.

**Claims**

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## WHAT IS CLAIMED IS:

1. A method of screening for compounds that reduce the bone deteriorating effects of glucocorticoids, comprising the steps of:

(a) contacting osteoblast and osteocyte cells with either a glucocorticoid alone or said glucocorticoid in combination with a test compound; and

(b) comparing the number of osteoblast and osteocyte cells undergoing apoptosis following treatment with said glucocorticoid alone or following treatment with said glucocorticoid in combination with said test compound, wherein a lower number of apoptotic cells following treatment with said glucocorticoid in combination with said test compound than with said glucocorticoid alone indicates that the test compound reduces the bone deteriorating effects of said glucocorticoid.

2. The method of claim 1, wherein said contacting is selected from the group consisting of *in vitro* cell cultures and *in vivo* murine animal model.

3. The method of claim 1, wherein determination of said apoptosis is selected from the group consisting of TUNEL, DNA fragmentation and immunohistochemical analysis.

4. The method of claim 1, wherein said test compound has little effect on the anti-inflammatory properties of said glucocorticoid, further comprising the step of:



5 (c) comparing the anti-inflammatory response of said  
glucocorticoid in combination with said test compound to the anti-  
inflammatory response of said glucocorticoid alone, wherein  
10 essentially equivalent anti-inflammatory responses of said  
5 glucocorticoid alone and said glucocorticoid in combination with said  
test compound indicates that the test compound both reduces the  
bone deteriorating effects while retaining the anti-inflammatory  
15 properties of said glucocorticoid.

5. The method of claim 4, wherein said contacting is  
10 in an *in vivo* murine animal model.

6. The method of claim 4, wherein said anti-  
25 inflammatory response is determined by models of inflammation  
selected from the group consisting of the adjuvant-induced  
15 arthritis model and hindlimb inflammation model.

7. A method of screening for glucocorticoid analogs  
that possess decreased apoptotic properties towards osteoblast  
35 and osteocyte cells, comprising the steps of:

20 (a) contacting said cells with either a glucocorticoid  
or a glucocorticoid analog; and

40 (b) comparing the number of apoptotic cells  
following treatment with said glucocorticoid or said glucocorticoid  
analog, wherein a lower number of apoptotic cells following  
45 25 treatment with said glucocorticoid analog than with said  
glucocorticoid is indicative of a glucocorticoid analog that  
possesses decreased apoptotic properties towards said cells.

5                   8. The method of claim 7, wherein said contacting is  
selected from the group consisting of *in vitro* cell cultures and *in*  
10 *vivo* murine animal model.

5                   9. The method of claim 7, wherein determination of  
said apoptosis is selected from the group consisting of TUNEL, DNA  
15 fragmentation and immunohistochemical analysis.

10                   10. The method of claim 7, wherein said  
glucocorticoid analog retains anti-inflammatory properties, further  
20 comprising the step of:

25                   comparing the anti-inflammatory response of said  
glucocorticoid in combination with a test compound to the anti-  
inflammatory response of said glucocorticoid alone, wherein  
15 essentially equivalent anti-inflammatory responses of said  
glucocorticoid alone and said glucocorticoid in combination with said  
30 test compound indicates that the glucocorticoid analog possesses  
decreased apoptotic properties while retaining anti-inflammatory  
35 properties.

20                   11. The method of claim 10, wherein said contacting  
is in an *in vivo* murine animal model.

40                   12. The method of claim 11, wherein said anti-  
inflammatory response is determined by models of inflammation  
45                   25 selected from the group consisting of the adjuvant-induced  
arthritis model and hindlimb inflammation model.

5 13. A method of screening for compounds that stimulate bone development, comprising the steps of:

10 (a) contacting osteoblast and osteocyte cells with either a glucocorticoid or a test compound; and

5 (b) comparing the number of said cells undergoing apoptosis following treatment with said glucocorticoid and said test compound, wherein a lower number of apoptotic cells  
15 following treatment with said test compound than with said glucocorticoid is indicative of a compound that stimulates bone  
20 development.

14. The method of claim 13, wherein said contacting  
25 is selected from the group consisting of *in vitro* cell cultures and *in vivo* murine animal model.

15 15. The method of claim 13, wherein determination of said apoptosis is selected from the group consisting of TUNEL, DNA fragmentation and immunohistochemical analysis.  
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20 16. A method of screening for compounds that increase bone mineral density, comprising the steps of:

40 (a) contacting osteoblast and osteocyte cells with either a glucocorticoid or a test compound; and

45 (b) comparing the number of said cells undergoing apoptosis following treatment with said glucocorticoid and said  
25 test compound, wherein a lower number of apoptotic cells following treatment with said test compound than with said  
50

5 glucocorticoid is indicative of a test compound that increases bone mineral density.

10 17. The method of claim 16, wherein said contacting is selected from the group consisting of *in vitro* cell cultures and *in vivo* murine animal model.

15 18. The method of claim 16, wherein determination of said apoptosis is selected from the group consisting of TUNEL, DNA fragmentation and immunohistochemical analysis.

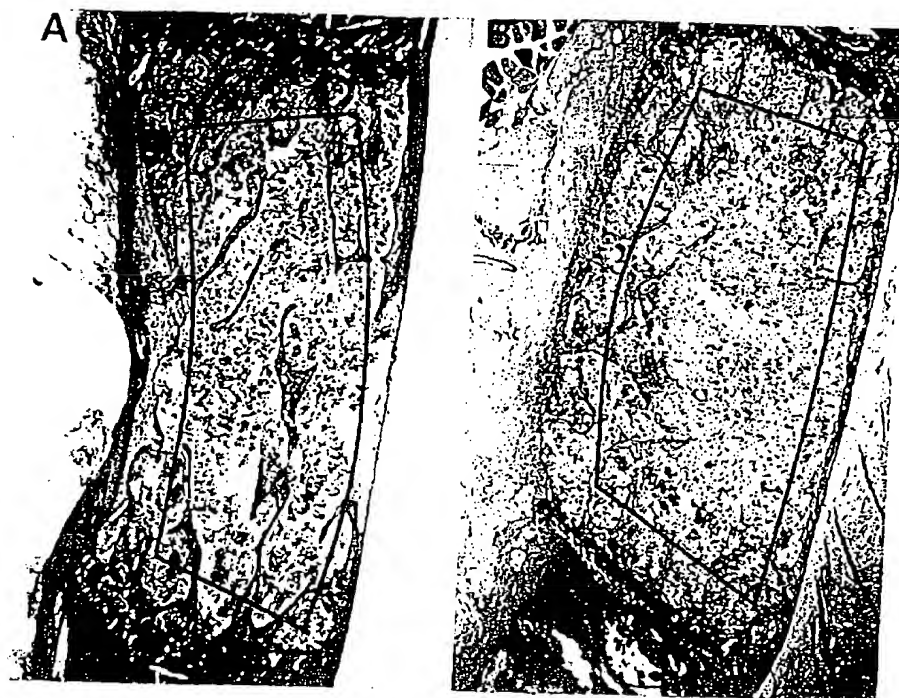


FIGURE 1

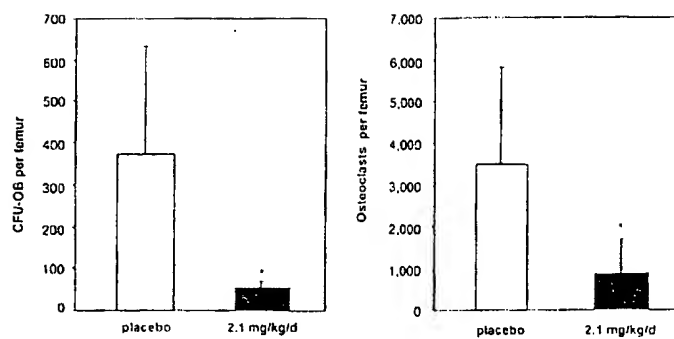


FIGURE 2

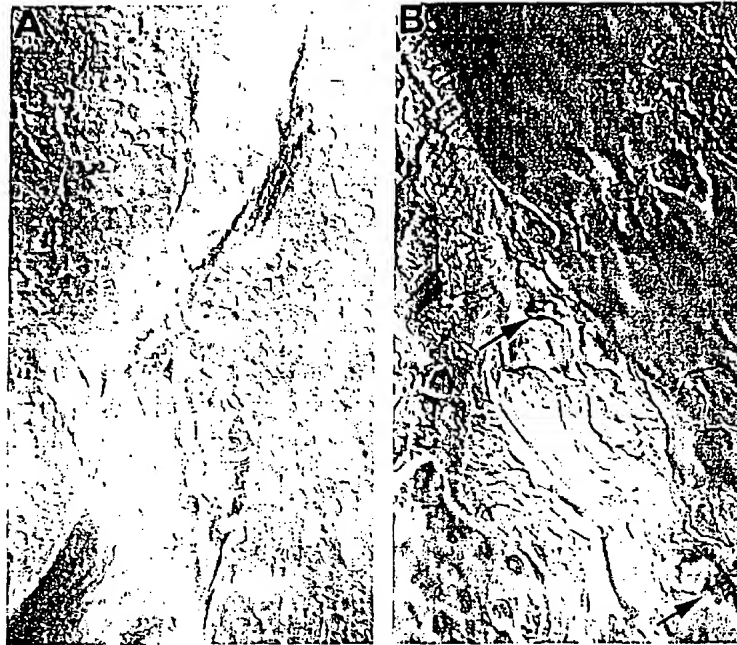


FIGURE 3

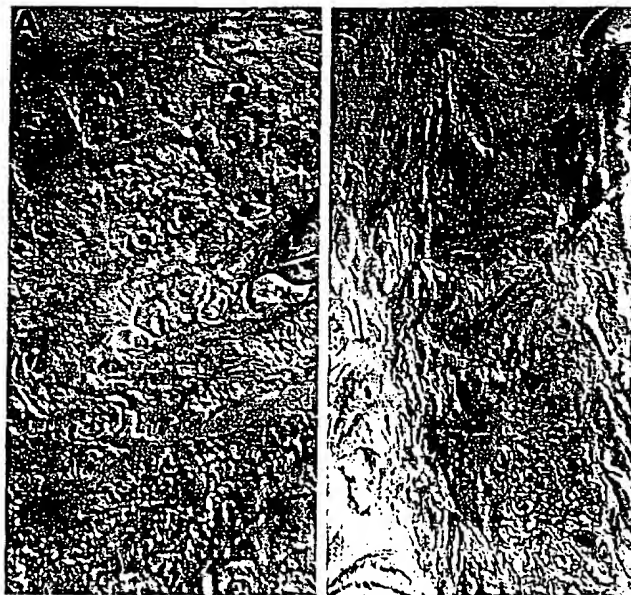


FIGURE 4



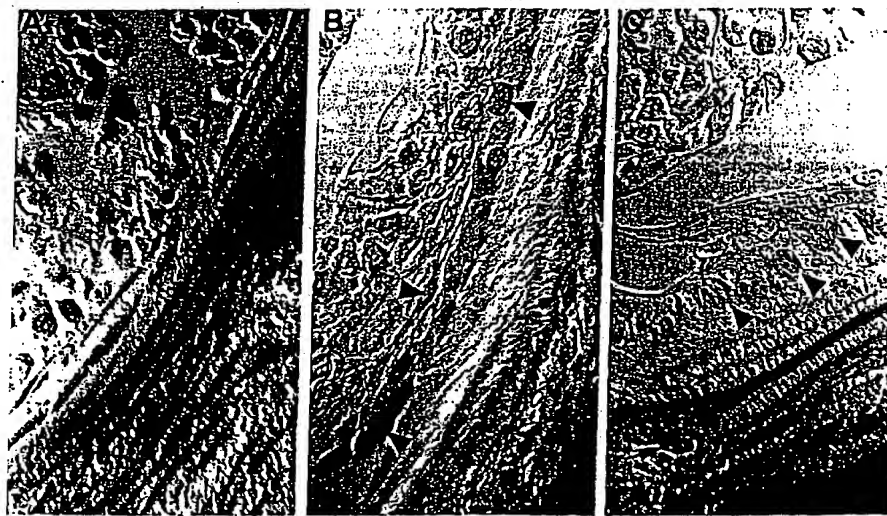


FIGURE 5

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/23395

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) :C12Q 1/00, 1/02; A61K 49/00; A61P 19/10 US CL :435/4; 424/9.2 According to International Patent Classification (IPC) or to both national classification and IPC																				
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/4; 424/9.2  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.																				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>																				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
X --- Y	ASMBR 19th Annual Meeting, page S455, abstract number S411, JILKA et al. 'Dexamethasone Promotes Apoptosis Of Osteoblast Progenitors In Murine Bone Marrow Cultures: Antagonism By IL-6 Type Cytokines,' Journal of Bone and Mineral Research, August 1997, Vol. 12 (Supp.).	1-3, 7-9, 13-18 --- 4-6, 10-12																		
X, P ----- Y, P	ASMBR-IBMS Second Joint Meeting, page S518, abstract number F458, BELLIDO et al. 'PTH Prevents Glucocorticoid Apoptosis Of Osteoblasts And Osteocytes In Vitro: Direct Interference With A Private Death Pathway Upstream From Caspase-3,' Bone, November 1998, Vol. 23, No. 5 (Supp.).	1-3, 7-9, 13-18 --- 4-6, 10-12																		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																				
<table border="0"><tr><td>* Special categories of cited documents:</td><td>"T"</td><td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td></tr><tr><td>"A" document defining the general state of the art which is not considered to be of particular relevance</td><td>"X"</td><td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td></tr><tr><td>"E" earlier document published on or after the international filing date</td><td>"Y"</td><td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td></tr><tr><td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td><td>"&amp;"</td><td>document member of the same patent family</td></tr><tr><td>"O" document referring to an oral disclosure, use, exhibition or other means</td><td></td><td></td></tr><tr><td>"P" document published prior to the international filing date but later than the priority date claimed</td><td></td><td></td></tr></table>			* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means			"P" document published prior to the international filing date but later than the priority date claimed		
* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																		
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"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																		
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"O" document referring to an oral disclosure, use, exhibition or other means																				
"P" document published prior to the international filing date but later than the priority date claimed																				
Date of the actual completion of the international search 23 DECEMBER 1999		Date of mailing of the international search report <b>14 FEB 2000</b>																		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>Peter Brunovskis</i> PETER BRUNOVSKIS, PH.D. Telephone No. (703) 308-0196																		

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/23395

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEINSTEIN et al. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids: potential mechanisms of their deleterious effects on bone. Journal of Clinical Investigation. July 1998. Vol. 102, No. 2, pages 274-282, see entire document	1-18
Y	ROGERS et al. Bisphosphonates induce apoptosis in mouse macrophage-like cells in vitro by a nitric oxide-independent mechanism. Journal of Bone and Mineral Research. 1996, Vol. 11, No. 10, pages 1482-1491, especially pages 1482, 1489-1490 and references therein.	1-18
Y	GIULIANI et al. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures in vitro and promote early osteoblastogenesis in young and aged mice in vivo. Bone. May 1998, Vol. 22, No. 5, pages 455-461, see entire document.	1-18
Y	JILKA et al. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. Journal of Bone and Mineral Research. May 1998, Vol. 13, No. 5, pages 793-802, see entire document.	1-18
Y	TOMKINSON et al. The role of estrogen in the control of rat osteocyte apoptosis. Journal of Bone and Mineral Research. August 1998, Vol. 13, No. 8, pages 1243-1250, see entire document.	1-18
Y	HILL et al. Multiple extracellular signals promote osteoblast survival and apoptosis. Endocrinology. 1997, Vol. 138, No. 9, pages 3849-3858, see entire document.	1-18
X, P -----	MANOLAGAS et al. New developments in the pathogenesis and treatment of steroid-induced osteoporosis. Journal of Bone and Mineral Research. July 1999, Vol. 14, No. 7, pages 1061-1066, see entire document.	1-3, 7-9, 13-18 -----
Y, P		4-6, 10-12

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/23395

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN, MEDLINE, CAPLUS, BIOSIS, EMBASE, SCISEARCH, WEST, USPT, EPAB, JPAB, DWPI, TDBD

search terms: apoptosis, tunel, dna fragmentation, osteoblast, osteocyte, glucocorticoid, bone, osteoporosis, manolagas